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Structure of the MazF-mt9 toxin, a tRNA-specific endonuclease from *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis (TB) is a severe disease caused by *Mycobacterium tuberculosis* (*M. tb*) and the well-characterized *M. tb* MazE/F proteins play important roles in stress adaptation. Recently, the MazF-mt9 toxin has been found to display endonuclease activities towards tRNAs but the mechanism is unknown. We hereby present the crystal structure of apo-MazF-mt9. The enzyme recognizes $tRNA^{Lys}$ with a central UUU motif within the anticodon loop, but is insensitive to the sequence context outside of the loop. Based on our crystallographic and biochemical studies, we identified key residues for catalysis and proposed the potential tRNA-binding site.

1. Introduction

Tuberculosis (TB) is one of the most severe diseases, caused by *Mycobacterium tuberculosis* (*M. tb*). TB infects one third of the population in the world, and its fatality rate is only second to acquired immune deficiency syndrome (AIDS) in all the infectious diseases [1]. Therefore it poses a serious threat to human health.

Toxin-antitoxin (TA) systems have been widely discovered in prokaryotic organisms [2,3]. Their functions include stabilizing bacterial genomes, influencing the formation of bacterial biofilms, development of bacterial persistent infection, and formation of bacterial resistance to phage infection [4,5]. A TA system generally consists of two ingredients, a stable toxic protein and an unstable antitoxin, which can either be a protein or RNA molecule [6]. In terms of the characteristics and action modes of the antitoxin molecules, TA systems fall into six distinct types [7]. The *Escherichia coli* MazEF toxin-antitoxin was the first and best-characterized TA system. The two proteins are encoded by *mazE* and *mazF* respectively, two adjacent genes in the chromosome. MazF cleaves mRNA sequences containing the ACA motif and thus suppresses protein synthesis [8,9]. The structure of *Escherichia coli* MazF (EcMazF) in complex with MazE was determined in 2003 (PDB 1UB4) [10]. MazE and MazF form a linear MazF2-MazE2-MazF2 heterohexamer, where the MazE homodimer sits in the middle and interacts with the flanking MazF homodimers on each side, using their protruding C-terminal tails. Following this work, quite a few structures of EcMazF and mutants were also solved (PDBs 3NFC, 5CQY, 5CQX, 5CO7, 5CKH, 5CKF, 5CKE, 5CKD, 5CKB, and 5CK9), including nucleic acid-bound forms [11]. Generally, MazF proteins tend to form homodimers. They adopt a classic form of MazF/CcdB fold, consisting of five β-strands and a C-terminal α-helix. In addition, MazF from *Bacillus subtilis* (BsMazF) and *Staphylococcus aureus* (SaMazF), in the apo form or in complex with their nucleic acid substrates have been determined, which will not be detailed here (PDBs 4MDX, 4ME7, 4MZM, 4MZP, and 4MZT) [12,13].

Currently, more than 80 members of *M. tb* TA systems have been identified [3,14]. As opposed to most prokaryotes, which mostly have one or two MazEF TAs, *M. tb* has nine such systems [3,14]. Most of the MazF members are ribonucleases, acting on mRNA or rRNA substrates. In 2016, Schifano et al. found that $tRNA^{Pro(GGG)}$ and

Abbreviations: AIDS, acquired immune deficiency syndrome; BL17U, beamline 17U; *M. tb*, *Mycobacterium tuberculosis*; PSP, PreScission protease; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; Ni-NTA, nickel-nitrilotriacetic acid; SSRF, Shanghai Synchrotron Radiation Facility; TA systems, toxin-antitoxin systems; TB, Tuberculosis; TF, trigger factor; WHO, World Health Organization; EcMazF, *Escherichia coli* MazF; BsMazF, *Bacillus subtilis* MazF; SaMazF, *Staphylococcus aureus* MazF.

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$tRNA^{Lys(UUU)}$ were the principal targets of MazF-mt9, and the tRNAs were specifically cleaved at a UUU motif in either the D-loop or the anticodon loop. Additionally, the cleavage depends on both the shape and the sequences of tRNA [15]. tRNAs were known to be dissected by the VapC family members [16], another toxin family in *M. tb*. MazF-mt9 represents the first and the only MazF member to specifically degrade tRNA, and therefore the molecular mechanism of this activity is of great interests. Additionally, the structural information of all nine toxins of *M. tb* is lacking. The structures of MazF-mt1, MazF-mt3 and their variants in complex with RNA or DNA were reported only quite recently (PDBs 5HJZ, 5HK0, 5HK3 and 5HKC), but the related work has not been published yet.

In this study, we determined the crystal structure of MazF-mt9 in its apo form. We investigated the biochemical properties of MazF and its substrate requirement. By sequence alignment and structure analyses, we identified key residues and proposed their roles in catalysis. Lastly, structural analysis revealed a highly positively charged dimer interface formed by MazF and we proposed that this deeper and wider interface may serve as the structural basis for MazF to specifically recognize and cleave tRNA substrates. Our work is among the first few structural studies on *M. tb* MazFs, and the structure presented here accounts for the substrate preference of MazF-mt9.

2. Materials and methods

2.1. Cloning, expression, and purification of MazF-mt9 and mutants

The gene encoding *M. tb* MazF-mt9 (Rv2063) was amplified by PCR from the genomic DNA from the *M. tb* H37Rv strain (ATCC Number 256181/H37Rv) with the primers 5′-AGCAGGCGGAGCTCTTGGCTGAGCCACGGCGA-3’ (forward) and 5′-TCAAGCTTGTCGACTCACGGGTCCCGGCCACC-3’ (reverse). After the double digestion by the *Sac*I and *Sal*I restriction enzymes, the PCR product was ligated into a modified pCold TF vector (TaKaRa). In order to efficiently cleave off the N-terminal trigger factor (TF) fusion protein, 6 × Asn residues were inserted between TF and the PreScission protease (PSP) site. The mutants of full-length MazF were generated by the *QuikChange* method (Stratagene) using the wild type (WT) as the template.

The plasmids encoding the WT or mutants of *M. tb* MazF-mt9 were transformed into *E. coli* strain BL21 (DE3) for overexpression. The cells were cultured overnight in Luria-Bertani broth containing 50 μg/ml ampicillin at 37 °C. 2-L fresh culture medium was then inoculated with 5-ml overnight culture. When the OD_{600} value reached 0.6–0.8, the cells were induced for overnight at 18 °C with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The purification of the fusion proteins involves a two-step strategy comprising the Ni-NTA affinity and the QHP anion exchange chromatography. The MazF-mt9 protein and mutants were obtained by collecting the unbound fractions from the Ni-NTA affinity column after digestion with the PreScission protease overnight. The proteins of interests were finally passed through the Superdex 200 size-exclusion column (GE Healthcare), and concentrated to 3 mg/mL before storage.

2.2. Crystallization, data collection, and structure determination

Initial crystallization screening was set up by a Mosquito crystallization robot (TTP Labtech) using the sitting-drop vapor-diffusion method in 96-well plates at room temperature. Hits were obtained overnight and diamond-shaped crystals were crystallized under a condition of 1.5 M NaCl and 0.1 M NaOAc.

A native 2.35 Å diffraction dataset of MazF-mt9 was collected using the beamline 17U (BL17U1) at the Shanghai Synchrotron Radiation Facility (SSRF, Shanghai, P.R.China) and was processed with the program *HKL2000* [17]. The structure was solved by molecular replacement using the program *PHENIX* with the coordinates of EcMazF structure (PDB 5CK9) [11] as the search model. The initial solution was built manually according to the electron density map with *COOT* [18]. Multiple cycles of refinement alternating with model rebuilding was carried out by *PHENIX. refine* [19]. The final model represents 81.4% of the full-length protein, and was validated by *SFCHECK* [20]. The structural figures were produced with *PyMOL* (www.pymol.org). All data collection and refinement statistics are presented in Table S1.

2.3. In-vitro cleavage assays

The preparation of the tRNA transcripts was described in a previous protocol [21]. The in-vitro tRNA cleavage assay followed the protocol described by Winther and Gerdes [22] with slight modifications. The assay mixture contains 20 mM HEPES (pH 7.5), 50 mM potassium chloride, 1 mM DTT and 0.8 μM *M. tb* $tRNA^{Lys(UUU)}$ or $tRNA^{Gln(UUG)}$ substrates. 0.4 μM MazF-mt9 WT or mutants were added to initiate the reactions, which was incubated at 37 °C. The reactions were stopped after 30 min by adding 2 × formamide gel-loading buffer (95% w/v formamide, 50 mM EDTA). The samples were denatured at 95 °C for 5 min prior to electrophoresis in a 15% Urea-PAGE gel containing 7 M urea, followed by ethidium-bromide staining. To study the effects of pHs on the enzymatic activity, the pH buffers (20 mM) in the assay mixture were changed from 5.5 to 11 as indicated.

3. Results and discussion

3.1. Overall structure of MazF and structural homologs

The cloning of WT MazF into the pColdTF vector with the fusion to an N-terminal TF protein allows overproduction of the target protein. After the cleavage of the fusion partner TF by PSP, we obtained the MazF protein in large quantities with high purity. The full-length protein with the preceding GPEL tetrapeptide (from the protease recognition site) was subsequently crystallized.

In the crystals of the apoprotein, each asymmetric unit contains two monomers (Fig. 1A). Chain A is visible from Ala2 to Pro114, while chain B is visible from Met1 to Pro114. Both chains are disordered in the N-, C-termini, and the internal Gly15-Pro22 fragment is also unstructured. The two monomers in the asymmetric unit form a dimer in a shoulder-to-shoulder fashion, using three small loops (Val31-Thr38, Glu43-Val46, Val76-Ala82) and the C-termini (Leu107-Pro114) as the dimer interface and burying 2115 $Å^2$ surface area. The two monomers resemble each other, and the superposition of the two chains yielded an RMSD of ~0.5 Å for 106 Cα atoms. Each monomer features a central antiparallel five-strand β-sheet with additional five helices. The C-terminus of each subunit forms a major helix, and it partially contributes to the formation of the dimer. The topology of the dimer is illustrated in Fig. 1B.

Dali search for structurally similar proteins [23] resulted in several orthologs from different organisms, and the three most similar proteins are the *M. tb* toxin (PDB 5CCA), *E. coli* MazF/E24A (PDB 5CKF) [11], *E. coli* MazF in complex with its antitoxin MazE (PDB 1UB4) [10], and *Vibrio fischeri* CcdB toxin (PDB 3JRZ) [24] (Fig. 1C). Among these proteins, the closest structural homolog *M. tb* toxin can be superimposed onto MazF-mt9 with an RMSD of 1.31 Å for 94 Cα atoms, suggesting relative large variations between the two structures. By overlaying these structures, we found that the core nuclease domain is preserved among these proteins (Fig. 1C). Additionally, the multiple sequence alignment indicated

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